Membranous nephropathy: recent travels and new roads ahead

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Insights from experimental studies have been recently translated into substantial advances in understanding the pathogenesis of human membranous nephropathy (MN). These include identification of neutral endopeptidase (NEP) as the target antigen in alloimmune MN resulting from fetomaternal immunization in NEP-deficient mothers, and our demonstration that a high proportion of patients with idiopathic MN (IMN) have circulating antibodies to the M-type phospholipase A2 receptor (PLA2R), a transmembrane protein located on podocytes. Here we highlight the studies that led to these discoveries and our current knowledge about the possible role of anti-PLA2R autoantibodies in the pathogenesis of IMN. Given that the sensitivity and specificity of anti-PLA2R for IMN are >75 and 100%, respectively, we foresee that a widely available assay for anti-PLA2R will prove to be valuable for diagnosing IMN, distinguishing it from secondary MN, and evaluating response to therapy. We suggest reasons why 25% of patients with IMN have tested negative for anti-PLA2R, and propose possible explanations for the presence of complement deposits in IMN despite the fact that immunoglobulin G4 (IgG4), the predominant anti-PLA2R IgG subclass, is incapable of activating the classical complement pathway. Finally, we point out avenues to be explored, including the events that induce production of anti-PLA2R, their ability to cause podocyte injury, the role of complement, and the nature of the antibodies in secondary forms of MN.

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Membranous nephropathy (MN), despite its recognition as one of the leading causes of idiopathic nephrotic syndrome, remains a relatively rare disease. Why then, has it garnered so much attention in the nephrology community over the last several decades? The answer, in part, is that idiopathic MN is a conceptually simple organ-specific autoimmune disease with pronounced direct and secondary effects. Through studies detailed below, it has become clear that circulating autoantibodies bind to target antigens on the podocyte foot process to initiate the disease process. There, the deposited antibody–antigen complexes not only give rise to the microscopic features by which the disease is characterized but also activate the complement system, leading to injury to the podocyte and subsequent proteinuria. This review will serve to highlight the most recent additions to this ever-evolving field of research.

WHAT DEFINES MN?

The features that characterize MN as a histopathological entity result from the immune deposits that form at the base of the foot processes of the glomerular visceral epithelial cell or podocyte. The name membranous derives from the thickened glomerular basement membrane (GBM) that is often evident by light microscopy in later stages of the disease. The immune deposits and, more important, the additional matrix material laid down by the injured podocytes are responsible for thickening of the GBM as the disease progresses. Using a silver methenamine stain, David Jones in 1957 first described argyrophilic 'spikes' or 'clubs' that represent this new matrix material between and around the unstained immune deposits.[1] In contrast, immunofluorescence for total immunoglobulin G (IgG) or IgG4 highlights the deposits themselves, yielding fine granular staining in a capillary loop pattern. Electron microscopy details the amorphous, electron-dense deposits that occur in a subepithelial and intramembranous position, as well as the effacement of the podocyte foot processes and other signs of podocyte injury.

SECONDARY FORMS OF MN

Most cases of MN in developed countries such as the United States are idiopathic MN (IMN); however, a number of secondary processes can also cause MN that is clinically and histologically similar to IMN. Worldwide, chronic infections

such as hepatitis B, malaria, syphilis, and schistosomiasis are the most important causes of secondary MN. Systemic lupus erythematosus can give rise to a membranous form of glomerular disease, classified as class V lupus nephritis. Other autoimmune diseases such as rheumatoid arthritis, autoimmune thyroid diseases, and Sjögren's syndrome can all be associated with MN. Historically, certain medications used for the treatment of rheumatoid arthritis such as gold salts, penicillamine, and some NSAIDs were causally linked to MN. Solid tumors are associated with secondary MN more often than chance alone would predict,[2] and on rare occasions remissions and relapses of the glomerular disease have been noted to occur with removal or relapse of the malignancy. Finally, MN can occur *de novo* after renal transplantation or allogeneic hematopoietic stem cell transplantation, perhaps reflecting alloimmunization to a minor histocompatibility antigen expressed in the glomerulus.[3,4]

Secondary forms of MN often exhibit histopathological clues that distinguish them from IMN, although this is not always the case. As opposed to the exclusively subepithelial and intramembranous deposits seen in IMN, secondary forms, especially membranous lupus nephritis, often have mesangial and subendothelial deposits. Tubuloreticular inclusions may also been seen within the glomerular endothelium on electron microscopy in lupus-associated MN. The IgG subclasses found within the glomerular deposits also differ. In contrast to the predominant IgG4 found in IMN, IgG2 and IgG3 are typically most abundant in secondary (lupus- and malignancy-associated) forms of MN.[5–7] Finally, the nature of the electron dense material itself may herald a secondary cause. A form of MN characterized by spherular structures within the subepithelial deposits has been described that appears to be distinct from its idiopathic cousin.[8]

WHAT HAVE WE LEARNED FROM HEYMANN NEPHRITIS?

An experimental rat model developed by Walter Heymann in the late 1950s has provided much of our knowledge about the pathophysiological mechanisms of human MN.[9] Known as Heymann nephritis (HN), the model is induced by the active or passive immunization of rats with fraction 1A (Fx1A), a mixture of antigens derived from rat proximal tubular brush border. These rats develop a proteinuric glomerulopathy with histological features virtually identical to the human disease. The model and its findings have been well reviewed in detail elsewhere;[10] however, several key findings that have helped to establish the pathogenetic paradigm in MN need special emphasis. There was initial debate as to the source of the subepithelial deposits, and the prevailing early view was that they represented circulating immune complexes that deposited in the glomerulus. Others speculated that freely circulating antigens were initially 'planted' in the GBM, followed later by antibody binding. Definitive experiments using *in vitro* and *ex vivo* perfused rat kidneys in a single-pass system showed that the antibodies bind *in situ* to a target antigen intrinsic to the glomerular capillary wall.[11–13] The primary antigen targeted in HN was ultimately identified as the transmembrane protein megalin, an endocytic receptor in the low-density lipoprotein receptor family that is located within coated pits on rat podocyte foot processes and proximal tubular brush borders.[14–18]

Another key finding in the HN model is that the development of proteinuria in most strains of rats is critically dependent on complement activation within the glomerular immune deposits. Rats depleted of complement with cobra venom factor do not develop proteinuria when injected with anti-Fx1A,[19,20] and deficiency or depletion of the terminal complement components C6 and C8 precludes assembly of the membrane attack complex C5b-9 and prevents podocyte injury and proteinuria, despite formation of subepithelial immune deposits.[21–23] These and subsequent findings in HN established that complement-fixing anti-megalin antibodies aggregate the antigen and activate complement leading to C5b-9-induced sublethal podocyte injury facilitated by accompanying antibodies that abrogate the effect of local complement-regulatory proteins.[24] The injured podocytes undergo a number of cytopathological changes, including the generation of reactive oxygen species and eicosanoids, reorganization of the actin cytoskeleton, and dissociation of slit diaphragm complexes.[25]

Although the active and passive HN models have been invaluable in defining pathogenic mechanisms in MN, they do have certain limitations, some of which have called into question HN's relevance to the human disease. First and most important is the fact that megalin is not expressed on human podocytes.[26] Second, attempts by several investigators failed to disclose a similar antigen in human MN. Third, the pathogenic IgG antibodies in HN are clearly able to fix complement, whereas IgG4, the predominant antibody in human MN, is believed to be an anti-inflammatory Ig incapable of activating the classical pathway of complement.[27] Fourth, although complement factors C3 and C5b-9 are found in the same fine granular pattern as IgG in MN biopsies,[28] and the C5b-9 complex has been found in the urine of such patients,[29,30] it is important to note that there are several animal models in which antibodies directed at podocyte antigens cause proteinuria in the absence of complement,[31–33] which suggests a direct cytopathic effect.

ANTENATAL ALLOIMMUNE MN

The first and best evidence that the paradigm of *in situ* immune complex formation also applies to human MN was established by Debiec *et al.*[34] in a rare form of neonatal MN. In a single case report, followed later by several more cases,[35] they described an infant born with the nephrotic syndrome, the cause of which was found to be MN on renal biopsy. Investigation into the cause revealed that the mother was genetically deficient in neutral endopeptidase (NEP), and was immunized to this protein during a previous miscarried pregnancy from an NEP-positive father. In her subsequent term pregnancy, circulating anti-NEP antibodies crossed the placenta as well as the fetal GBM, bound NEP on the fetal podocytes, and instigated a similar disease process as that described in the experimental model. Two important features should be noted in these cases of alloimmune antenatal MN.

First, the disease resolved in the infant once the maternal anti-NEP antibody was cleared from its system. Second, proteinuria occurred only in the children of mothers who had both IgG1 and IgG4 anti-NEP.[35] A mother who was found to have only the IgG4 subclass of anti-NEP did not give birth to an affected infant. As IgG4 is unable to activate the classical complement system, this suggests the need for complement fixing IgG1 or IgG3 to cause clinically evident disease.

IDENTIFICATION OF PLA2R AS TARGET ANTIGEN

Our laboratory has been involved in the search for the human MN antigen, and similar to others, we approached the problem by using sera from individuals with MN to screen human glomerular proteins by immunoblot analysis. We soon realized that a number of these sera contained antibodies that specifically recognized a high-molecular-weight glycoprotein in normal human glomeruli. This protein was detected only in the absence of reducing agents, suggesting the presence of one or more epitopes in the molecule whose conformation was dependent on disulfide bonds and providing a potential explanation as to why other laboratories had not identified this protein using similar techniques. Capitalizing on several properties of this glycoprotein and with mass spectrometric analysis, we ultimately identified the protein reactive with these antibodies as the M-type phospholipase A2 receptor (PLA2R).[36] This protein, also expressed in lung and on neutrophils,[37,38] appears largely restricted in the kidney to the podocyte, in which it is abundantly expressed.[36] Autoantibodies to PLA2R are largely IgG4, the least abundant IgG subclass in general, but known to be the predominant subclass in IMN immune deposits. In confirmation of this latter point, IgG4 could be colocalized with the PLA2R antigen within the immune deposits of IMN biopsy samples, and anti-PLA2R IgG could be eluted from biopsy samples of patients with IMN, but not lupus-associated MN or IgA nephropathy. All anti-PLA2R autoantibodies tested to date share the same property of detecting PLA2R only in its non-reduced state (LH Beck and DJ Salant, unpublished data).

PLA2R is an intriguing molecule, with properties that may ultimately prove important for its proposed role as target antigen in MN. It is a member of the mannose receptor family of proteins, which includes the mannose receptor, Endo180, DEC205, and the avian IgY receptor.[39,40] Three of these members have been shown to undergo a conformational change, forming an N-terminal hairpin bend in response to changes in pH or in association with ligand binding.[41–43] Furthermore, all undergo constitutive endocytic recycling at the plasma membrane,[44] which could provide a constant source of PLA2R at the podocyte foot process. We do not yet know whether the anti-PLA2R antibodies interfere with the normal function of PLA2R in podocytes, and the biological role of the protein in the kidney is unknown. PLA2R has recently been reported to promote replicative senescence in human fibroblasts, in part, by causing reactive oxygen species production and DNA damage.[45] It has also been shown to activate cytosolic PLA2 leading to membrane phospholipid hydrolysis and eicosanoid production.[46] Similar processes have been observed as downstream processes in the HN model,[25] and it will be of interest to see if such effects occur in human disease, mediated through PLA2R. A transcript encoding a form of human PLA2R that lacks the transmembrane and intracellular domains has been described,[47] but we have not detected this putative soluble form in normal or MN serum.

Current sensitivity and specificity analyses show that anti-PLA2R autoantibodies are present in greater than 75% of individuals with IMN, but never in those with secondary causes of MN, other glomerular or autoimmune diseases or normal controls. There may be several reasons that the sensitivity is not 100%, including potential technical issues with the immunoblot assay, additional antigens targeted in the disease, or misclassification of patients with IMN (who may have an unrecognized secondary cause). However, our leading hypothesis as to why there remain 25% of IMN patients who do not have anti-PLA2R antibodies is that there was an absence of immunologic disease activity at the time when their serum was sampled, despite proteinuria. We have shown presence of anti-PLA2R in patients initially nephrotic from IMN, an absence in many of those that undergo complete remission, and a recurrence of the autoantibodies in cases of relapse. However, for reasons explained below, the waters become muddier in those patients with subnephrotic proteinuria who have achieved a partial remission.

It is important to make this distinction between proteinuria, as a clinical marker of disease, and the presence of anti-PLA2R, a marker of immunologic activity in IMN. The two are closely, but not perfectly, related due to the time it takes to form and resorb the subepithelial deposits (see Figure 1). Protocol biopsies of renal allografts demonstrate that the earliest deposits that occur in recurrent MN can be associated with minimal proteinuria.[48] Conversely, proteinuria can remain after there is no further evidence of circulating autoantibodies. We have shown a linear correlation between anti-PLA2R and amount of proteinuria (LH Beck and DJ Salant, unpublished data), and note that at the point where autoantibody level reaches zero, there is on average still residual proteinuria, in the 2–3 g/day range. In patients who achieve complete remission in response to immunosuppressive therapy, anti-PLA2R levels become undetectable months before the proteinuria resolves completely. Although these findings could alternatively be explained by the continued circulation of additional unidentified pathogenic antibodies, they are perhaps more compatible with residual structural deficits in the absence of immunologic activity. In the experimental HN model, transplantation of an affected kidney to a naive host results in a significant decrease in proteinuria, but not its complete elimination.[49] In addition, GBM remodeling and sclerotic changes that occur in the glomerulus and interstitium in the face of longstanding MN can also cause persistent proteinuria unrelated to an ongoing immunologic process.

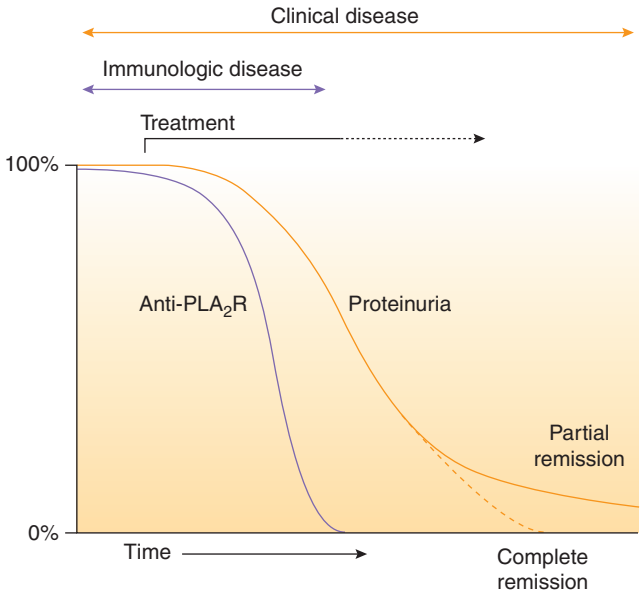

Figure 1 | Relationship between clinical disease (proteinuria) and immunological activity (circulating anti-PLA2R) in idiopathic membranous nephropathy.

POTENTIAL MECHANISMS OF PATHOGENESIS

Given the strong association of anti-PLA2R with disease activity, it appears likely that these autoantibodies have inherent pathogenicity. Proof, however, must await suitable transgenic animal models, as all small animals tested thus far do not express PLA2R on their podocytes, at least not in a form recognized by human autoantibodies. Further proof of pathogenicity might be forthcoming from studies of recurrent MN post-renal transplantation if it is found that persistence or reappearance of anti-PLA2R predates clinical or histological evidence of MN. Regarding potential mechanisms by which anti-PLA2R might cause podocyte damage, both complement-dependent and independent mechanisms are possible. As noted above, there is substantial evidence that complement is activated in the glomerular immune deposits in idiopathic MN; whereas, IgG4, the major IgG subclass present in the glomeruli as well as the predominant anti-PLA2R subclass, is generally regarded as being incapable of activating complement through the classical pathway. While it is possible that the smaller amounts of IgG1 anti-PLA2R detected in our studies may be responsible for activating the classical pathway—as reported in anti-NEP-induced alloimmune MN–most reports have failed to detect C1q deposits in idiopathic MN. This suggests the possibility that the alternative (AP) or mannan-binding lectin pathways may be involved. Of the two, the mannan-binding lectin pathway seems a better candidate because C4b, a product of mannan-binding lectin activation (but not the AP), is commonly found in idiopathic MN, whereas factor B of the AP is not.[50] In addition, mannan-binding lectin has been identified in the glomeruli in a high proportion of cases of idiopathic MN.[51]

Despite the well-documented presence of complement products in MN, it certainly is possible that the initial events involved in the pathogenesis of idiopathic MN are completely unrelated to complement activation. For instance, there are well-characterized cases in which human autoantibodies possess agonist activities. Examples include the thyroid-stimulating antibody in Grave's disease[52] and activating antibodies to the angiontensin II receptor that cause hypertension in pre-eclampsia and renal allograft rejection.[53,54] Anti-PLA2R antibodies may potentially interfere with the normal function of PLA2R, either as a false agonist or as an antagonist. An immediate answer to this question may not be possible, as the function of PLA2R in the podocyte is unknown at this point. However, the recent identification of PLA2R as a regulator of replicative senescence in human fibroblasts,[45] combined with the demonstration of markers of senescence in the podocytes in MN biopsies, [55] provides a plausible reason to investigate potential agonist activity of anti-PLA2R.

ARE THERE OTHER INTRINSIC GLOMERULAR ANTIGENS?

Might there be other podocyte antigens that could also incite MN? Although additional endogenous antigens might explain the fact that sera from 25% of patients with IMN do not react with PLA2R, this is not supported by our observations to date. We have found that sera from this patient population do not identify any other protein bands by immunoblot using extracts of normal human glomeruli. Other investigators have shown the presence of antibodies to other, mainly intracellular glomerular proteins.[56] Ronco and Debiec[57] have additionally reported low-level anti-NEP antibodies in adult patients with IMN. Future studies will be needed to see if they arise contemporaneously with or subsequent to anti-PLA2R antibodies, and if both are ultimately needed for pathogenesis.

We are largely unaware of the processes underlying the deposition of immune complexes in secondary forms of MN. Our immunoblot assay does not suggest the presence of a major intrinsic glomerular antigen. Other investigators have shown the presence of nucleosomes within the subepithelial deposits in lupus-associated MN.[58] Similarly, the hepatitis B e antigen (HBVe Ag) and tumor-associated markers have been demonstrated in these deposits.[59,60] It is not known whether their presence is pathogenic, or whether they are just nonspecifically trapped there. It has been hypothesized that cationic antigens (histones, HBVe Ag) readily cross the GBM and become planted in a subepithelial location, after which circulating antibodies bind to form immune complexes. Others have suggested that low-affinity immune complexes are trapped at the luminal surface of the GBM, dissociate, and then reform on the abluminal side to form subepithelial immune complexes. Clearly, secondary MN is a field awaiting further investigation with the molecular and proteomic tools now at hand (Table 1).

NEW ROADS AHEAD

Detection of circulating anti-PLA2R autoantibodies is likely to have a future role in diagnosis and monitoring of disease activity in IMN. The robust sensitivity and specificity of the test may afford patients with the nephrotic syndrome, a

Table 1 | Causes of idiopathic and secondary membranous nephropathy (MN)

Idiopathic
Anti-phospholipase A2 receptor (75%)
Antigen still unknown or simply inactive (25%)
Secondary (causative antigen still unknown)
Systemic lupus erythematosus
Hepatitis B
Malignancy
Other causes
Alloimmune
Fetomaternal alloimmunization to neutral endopeptidase
De novo MN post-renal transplantation (?)
MN post-allogeneic stem cell transplantation (?)

diagnosis of IMN without requiring an invasive kidney biopsy. Furthermore, in those who do receive a biopsy that reveals MN, an absence of anti-PLA2R may prompt a more thorough search for secondary causes. This distinction may also be made on the grounds of immunofluorescence of the biopsy tissue. In the secondary MN cases that we have tested, the PLA2R antigen did not colocalize with IgG in the immune deposits, but was rather still present in the podocyte cell body as in normal kidneys.

Assessing the presence of anti-PLA2R in patients with subnephrotic proteinuria after treatment for MN may also provide important information. Knowledge of whether this residual proteinuria represents structural changes that are best treated conservatively, or a smoldering immunological activity that requires further or different immunosuppressive therapy, would be helpful clinically. This information would also be valuable in selecting patients for clinical trials to avoid including subjects with immunologically inactive disease.

As yet we have no good explanation why MN is limited to the glomeruli given that PLA2R is also expressed in other organs. As it may simply have to do with access of the antibodies to the antigen across more or less permeable capillary beds, more subtle explanations related to molecular conformation or epitope masking are equally plausible. Finally, we have not even scratched the surface to uncover the initiating events that lead to the production of anti-PLA2R autoantibodies. The fact that all samples tested to date identify the same or closely related conformation-dependent epitope on PLA2R with little evidence of epitope spreading to the rest of the molecule may provide a clue if molecular modeling is possible. Molecular mimicry as demonstrated in antineutrophil cytoplasmic antibody-associated vasculitis[61] is an attractive possibility worthy of investigation once the anti-PLA2R epitope is better defined.

CONCLUSIONS

Fifty years after the introduction of the HN model, we are now poised to enter a new era in the investigation of human MN. Defining the pathogenic mechanisms by which anti-PLA2R autoantibodies act is certainly paramount to a better understanding and treatment options for this disease. The decades of work performed in the HN model will serve as a guide, although we expect many novel and interesting findings along the way. Despite our current ignorance about the precise pathomechanisms of anti-PLA2R, we anticipate that measurement of these autoantibodies will become a serologic test to diagnose IMN, to distinguish 'idiopathic' MN from secondary forms, and to monitor and tailor immunosuppressive therapy.

DISCLOSURE

LHB reports receiving grant support from Amgen; and DJS reports receiving grant support and consulting fees from Questcor Pharmaceuticals, and consulting fees from Taligen. Both authors are listed as inventors on a patent pending for a diagnostic immunoassay to detect anti-PLA2R antibodies in MN. No other potential conflict of interest relevant to this article was reported.

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